Nocturnal whey protein ingestion impairs postprandial glucose tolerance at breakfast

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Abstract

Poor postprandial glucose control is a risk factor for multiple health conditions. The second-meal effect refers to the progressively improved glycaemic control with repeated feedings, an effect which is achievable with protein ingestion at the initial eating occasion. The most pronounced glycaemic response each day therefore typically occurs following breakfast, so this study investigated whether ingesting protein during the night could improve glucose control at the first meal of the day. In a randomised cross-over design, fifteen adults (7 males, 8 females; age, 22 ± 3 years; BMI, 24.0 ± 2.8 $kg \cdot m^{-2}$; fasting blood glucose, 4.9 ± 0.5 $mmol \cdot L^{-1}$) woke at 0400 ± 1 h to ingest 300 ml water with or without 63 g whey protein. Participants then completed a mixed-macronutrient meal tolerance test (1 g carbohydrate·kg body mass^{-}1, 563 ± 104 kcal,) 5 h 39 min following the nocturnal feeding. Nocturnal protein ingestion *increased* the glycaemic response (incremental area under curve) to breakfast by 43.5 ± 55.5 mmol•120 min•L^{-1} (p=0.009, d=0.94). Consistent with this effect, individual peak blood glucose concentrations were 0.6 ± 1.0 $mmol \cdot L^{-1}$ higher following breakfast when protein had been ingested (p=0.049, d=0.50). Immediately prior to breakfast, rates of lipid oxidation were 0.02 ± 0.03 $g \cdot min^{-1}$ higher (p=0.045) in the protein condition, followed by an elevated postprandial energy expenditure (0.09 ± 0.12 $kcal \cdot min^{-1}$, p=0.018). Postprandial appetite and energy intake were similar between conditions. This study reveals a paradoxical second-meal phenomenon whereby nocturnal whey protein feeding *impaired* subsequent glucose tolerance, whilst increasing postprandial energy expenditure.

INTRODUCTION

The postprandial glycaemic response to a fixed carbohydrate load can be used to assess the proficiency of glucose control[1]. Repetitive and/or prolonged hyperglycaemic episodes are linked to a heightened risk of multiple health conditions including: type 2 diabetes mellitus, cardiovascular disease and ultimately premature mortality[2]. Ineffective postprandial glucose tolerance also impairs satiety perception and is thus linked to obesity, a key risk factor for type 2 diabetes[3; 4]. Even amongst normoglycemic individuals [5], those with poorer postprandial glycaemic control are at increased risk of developing type 2 diabetes and cardiovascular disease[6]. The prevalence of these morbidities is increasing and presents a large personal and socio-economic burden [3; 7]. Several clinical markers are available to diagnose defective glucose control; incremental area under the blood glucose concentration curve (iAUC) reflects the response to a glucose challenge and provides a more valid indication of glucose tolerance than basal/fasted measures alone [2; 8]. This is entirely understandable given that the time spent hyperglycaemic is a primary factor in the aetiology of diabetic complications[9; 10].

Beyond the internal validity of postprandial glycaemia as a measure of glucose control, the external validity of fasted (i.e. post-absorptive) measures is also more limited given that typical eating patterns in most societies mean that humans spend most or all waking hours in a fed-state (i.e. post-prandial)[9; 11]. Of even greater practical relevance is the response to sequential meals, as most meals are therefore consumed in an already postprandial state. The 'second-meal effect' describes how initial glucose ingestion improves glucose tolerance at a second eating occasion [12]. Whilst this phenomenon was first discovered with sequential oral glucose tolerance tests (OGTT), a more ecologically valid method considers the effect of the mixed-macronutrient meals. This has been most commonly examined in the literature as the magnitude of metabolic responses to lunch when breakfast is consumed, comparative to no breakfast[8]. Notably, this effect also promotes a more even glycaemic stability across the day, thus eliciting a potentially beneficial effect on satiety perception[13]. The second-meal effect is hypothesised to involve several interacting mechanisms, such as enhanced hepatic and peripheral insulin sensitivity, potentiated insulin secretion, and slowed gastric emptying following ingestion of a second meal[14].

Whereas the influence of carbohydrate (CHO) and fat on second-meal effects are relatively well understood (i.e. high-CHO, low-glycaemic index (GI) and low-fat breakfasts are effective in optimising the insulin secretion and glycaemic response to lunch[15; 16; 17; 18]), the efficacy of protein has received less research attention. Several studies have demonstrated that protein, specifically whey, consumed as a preload can elicit the second-meal effect [19; 20; 21]. This has been attributed to augmented insulin secretion and insulin priming of the muscle and liver, alongside delayed gastric

emptying of the second load [20; 21]. Protein ingestion has also been linked to appetite suppression and a reduction in subsequent energy intake[4; 20; 22], although other studies report no such effects [19; 23]. In addition, recent evidence suggests that a high-protein breakfast may be a more potent stimulator of the second-meal effect than a high-CHO breakfast[24; 25], although this possibility requires confirmation in young, normoglycaemic populations. Moreover, previous studies have only examined the carry-over effects between the established daily sequence of eating occasions (i.e. breakfast-lunch-dinner), yet a novel strategy would be to attenuate the profound first glycaemic response after waking using a model wherein breakfast serves as the second meal of the day.

The purpose of this study, therefore, was to examine the effect of nocturnal whey protein ingestion on the glucose response to breakfast, alongside any secondary effects on subsequent satiety and energy intake later in the day. Based on extant literature examining the effects of prior feeding during the morning on metabolic responses to lunch, we hypothesised that protein ingestion will attenuate the glycaemic response to breakfast and reduce subsequent appetite and energy intake.

EXPERIMENTAL METHODS

Approach to the research question

The innovative nature of this investigation stems from the unique combination of protein ingestion with nocturnal feeding. Whey protein was chosen in preference to alternative protein sources due to a shorter gastric emptying time, resulting in a more rapid development and greater magnitude of hyperaminoacidaemia and therefore a more rapid insulin release. This is consistent with the rationale for our stated hypothesis above, that an increased morning insulin requirement is due to a lack of residual insulin from a preceding meal[26]. As such, this novel strategy may reduce the insulin requirement to breakfast, consequently improving glucose control.

Whey also reduces subsequent food intake and appetite to a greater extent than casein, egg or soy[20; 26; 27]. A whey protein dose between 20-40 g can be effective in reducing appetite, while as little as 10 g is reported to stimulate the second-meal effect[20]. A large, but palatable dose of 63 g was used in this novel protocol to conclusively elucidate any effects on sequential glycaemic response and satiety (i.e. if effects are observed then that provides proof-of-principle and could warrant further examination of lower doses). This protein solution was made up with 300 ml of water instead of milk to avoid confounding effects of fat ingestion and to allow any effects of protein to be isolated.

The time of protein feeding was 0400 ± 1 h to ensure a post-absorptive state following dinner and to allow sufficient time before breakfast for the acute initial metabolic response to the ingested

protein to subside. The time of feeding resulted in, on average, a 5 h 39 min period between protein ingestion and breakfast, which aligns with the majority of studies investigating the second-meal effect using 3-6 h between sequential meals[13; 18; 28; 29]. Additionally, participants were instructed to be in bed, lights out, at 23:00, which would therefore result in a minimum of 5 h separating dinner and the nocturnal protein feed. A mixed macronutrient tolerance test was deemed more appropriate than an OGTT as this study focuses on the response to sequential meals to provide practical results applicable to daily living. A porridge breakfast aligns with this aim as a common breakfast meal, providing information on the ability to process a realistic glucose and fat challenge.

[Figure 1]

Participants

Fifteen individuals who self-identified as healthy volunteered to participate (7 males, 8 females; age, 22 ± 3 years; BMI, 24.0 ± 2.8 kg•m^{-2}; resting heart rate, 69 ± 15 bpm; resting mean arterial pressure, 72 ± 6 mmHg; fasting blood glucose, 4.9 ± 0.5 mmol•L^{-1}; sleep chronotype, 54 ± 7 (intermediate)). Participants were informed of the study objectives, requirements and any potential risks before written consent was obtained. Ethical approval was received from the University of Bath research ethics committee (SESHES-18R1-004). Exclusion criteria included allergy or intolerance to any of the breakfast constituents or any metabolic conditions which may have posed undue personal risk to the participant or introduced bias into the experiment.

Study design

This study employed a randomised crossover design, comprising two conditions; nocturnal ingestion of either a protein solution (PRO) or water (CON). The following morning participants visited the laboratory to complete a mixed-macronutrient meal tolerance test. Participants were not blinded to the condition as it would have been immediately apparent upon ingestion and to analyse subsequent food choices participants needed to be aware of their consumption as they would in free-living conditions. Trials were completed within a month for males, or within 4-7 days for females to ensure the phase of the menstrual cycle was consistent between trials in order to avoid large systematic differences in glucose control due to menstrual cycle phase[30]. All procedures were performed in accordance with the declaration of Helsinki.

Experimental procedures

Participants refrained from vigorous physical activity and replicated their diet the day prior to both laboratory visits. Compliance was confirmed verbally upon laboratory arrival. A schematic for the study protocol is shown in **Figure 2**. Prior to data collection, participants completed the Horne & Östberg Morningness-Eveningness Questionnaire[31], to assess sleep chronotype and establish habitual morning or evening preference. Participants were instructed to be in bed, lights out at 23:00, awaking at 07:00 and arriving in the laboratory the following morning at 09:00. Participants awoke at 04:00 to consume the relevant solution. Compliance was confirmed with a text message to a researcher at this time. Based on the questionnaire results, these time components were adjusted ± 1 h for morning or evening preference. This aimed to minimise disturbance to habitual sleep patterns due to the confounding effect on glucose tolerance[32].

Upon laboratory arrival the following morning, anthropometric (height and mass) and pre-breakfast resting measures were collected. Fingertip blood glucose concentration was recorded using an automatic glucose analyser (coefficient of variation 5.03%) (FreeStyle Optium, Abbott Laboratories Ltd, Berkshire, UK). Three 5 min expired gas samples were collected using Douglas bags (Hans Rudolph, MO, USA), which were analysed using a Servomex 1440 Gas Analyser (Servomex Group Ltd., UK) and the volume of expired air determined by evacuating the Douglas bag with a dry gas meter (Harvard Apparatus, Kent, UK). Heart rate and blood pressure (Omron M2 Compact blood pressure monitor, Omron Healthcare Co., Netherlands) were collected, alongside 100 mm visual analogue scales.

A standardised porridge breakfast was then consumed, followed by a 2 h resting period in the seated position with water consumption permitted (ad libitum). Blood glucose measurements were collected every 15 min for the first 60 min and then every 30 min until 120 min. Five min expired air samples were collected at 20, 40, 75 and 100 min. At 60 and 120 min appetite VAS were completed. Upon completion of the tolerance test participants were free to leave the laboratory and completed a food diary for the remainder of the day under free-living conditions.

[Figure 2]

159 ***Nutritional composition***

160 Experimental solutions were prepared by researchers and provided to participants the night
161 before the trial. The protein solution contained: 75 g whey protein powder (Myprotein, Northwich,
162 UK), resulting in an intake of 63 g protein (21 g protein per 25 g serving), 300 ml water and 0 kcal
163 vanilla flavouring drops to taste (Myprotein, Northwich, UK). An amino acid profile for the whey
164 protein used is depicted in **Figure 1.** The control solution was 300 ml water. Breakfast consisted of
165 29% porridge oats (Sainsbury's, UK), 67% whole milk (3.7/100 g fat; Sainsbury's, UK) and 4%
166 granulated sugar (Sainsbury's, UK). Porridge was provided in quantities to deliver 1 g CHO·kg body
167 mass^{-1}, obtained from 80% oats and 20% sugar. This resulted in an energy intake of 563 ± 104 kcal,
168 containing 18.0 ± 3.3 g protein and 72.4 ± 13.9 g CHO. The quantity was replicated for the second
169 trial, regardless of any minor change in body mass. Participants were instructed to consume the
170 porridge within 15 min to standardise effects of eating rate upon appetite hormones[33].

171

172 ***Statistical analysis***

173 All data are presented as mean ± standard deviation. Statistical analyses were performed using
174 the Statistical Package for the Social Sciences (IBM SPSS Statistics 25), with statistical significance
175 accepted at an alpha level of $p \leq 0.05$. A minimum sample size of 11 was calculated in order to detect
176 an effect size of 0.95 with 80% power (G*Power 3.1.9.4), based on the effect size in similar studies
177 in normoglycaemic individuals [24; 34]. Data from one female participant who volunteered for the study
178 were not included in the analysis because she experienced very poor sleep quantity in the control
179 condition (>2 SD below the mean), given the established negative effect of sleep deprivation on
180 glucose tolerance[32]. This is illustrated by her almost doubled blood glucose iAUC in the CON
181 condition (396 *versus* 195 mmol·120min·L^{-1}).

182 Blood glucose iAUC was calculated using the trapezoid method[35]. Rates of oxygen
183 utilisation and carbon dioxide production were used to calculate respiratory exchange ratio (RER)
184 and energy expenditure from expired gas samples[36]. Substrate oxidation rates were calculated in
185 accordance with the stoichiometric equations outlined by Frayn [37] assuming negligible protein
186 oxidation. Of the three resting gas samples, the average of those within a 100 kcal• day^{-1} agreement
187 in energy expenditure was taken to calculate pre-breakfast values of substrate utilisation, RER and
188 energy expenditure. If none of the three bags met this criterion, the lowest of the three samples was
189 considered the most reflective measure of resting metabolic rate (this was the case for 5 out of 27
190 bags). Scores from the appetite VAS were combined to give an average appetite score at each time
191 point according to the equation outlined by Gonzalez and Stevenson [8]. Regarding sleep VAS, a

192 difference >10 mm is deemed of clinical relevance and more meaningful than statistical
193 significance[38], and thus this criterion was used when examining intraindividual differences in VAS
194 scores between conditions.

195 A Shapiro-Wilk test was used to assess the normality of intraindividual differences between
196 conditions. Consequently, a paired *t*-test was applied to analyse normally distributed parametric data
197 and a Wilcoxon test used for non-parametric data. Order effects were examined with a paired *t* test
198 for iAUC between trial 1 and 2 alongside a two-way mixed-models ANOVA (treatment x sequence);
199 there was a 2.2% decrease in iAUC from trial 1 to 2 with no significant order effect (p=0.875) and no
200 treatment x sequence interaction (p=0.509, F=0.463). Pearson correlation coefficients were calculated
201 for the difference in iAUC between conditions and both body mass and sleep quality (calmness and
202 wakefulness). A two-way repeated measures ANOVA (treatment x time) was used to examine
203 differences in blood glucose response and appetite over time. The Greenhouse-Geiser correction was
204 applied for epsilon values <0.75 and the Huynd-Feldt correction applied for less severe asphericity.
205 Effect size was calculated using pooled standard deviation, with Cohen's thresholds applied[39].

206

207

208 **RESULTS**

209 ***Blood glucose concentration***

210 Plasma glucose concentrations increased more rapidly in PRO than in CON, reaching a 7.5%
211 higher peak (7.9 ± 1.4 *versus* 7.3 ± 0.9 mmol•L^{-1}, p=0.049, d=0.50, moderate effect; **Figure 3A**) and
212 then remaining higher than the CON trial until the final blood sample, such that there was a main
213 effect of treatment (p=0.037, F=5.392) but no treatment x time interaction (p=0.308, F=1.241; **Figure**
214 **4**). Accordingly, there was a 28.1% mean increase in postprandial blood glucose iAUC in the PRO
215 condition (161.8 ± 55.7 v 116.3 ± 39.2 mmol•120 min•L^{-1} (p=0.009, d=0.94; **Figure 3B**). Time spent
216 above the clinical threshold of 7.5 mmol•L^{-1} blood glucose concentration was longer in the PRO trial
217 for 9 out of 14 participants by an average of 8.2 ± 13.4 min (15.9 ± 17 v 7.7 ± 11.6 min, p=0.039,
218 d=0.56, moderate effect). Although not significant (p=0.206, d=0.48, small effect), on average there
219 was a 6.4 ± 18.3 min delay in time to peak glucose with PRO (37.5 ± 16.4 min) compared to CON
220 (31.1 ± 9.2 min).

221

222 [Figure 3]

[Figure 4]

Substrate utilisation, respiratory exchange ratio and energy expenditure

Lipid oxidation was 30.8% greater at pre-breakfast (following intervention but prior to the test meal) in the PRO trial compared to CON (p=0.045) (**Table 1**). There were no significant differences between conditions at pre-breakfast for RER, CHO oxidation, or energy expenditure. Mean responses over the 120 min postprandial period did not differ between conditions for RER, CHO oxidation, or lipid oxidation. Postprandial energy expenditure was greater in the PRO trial (p=0.018).

[Table 1]

Subjective appetite and energy intake

There were no significant differences in any appetite ratings or combined appetite score at pre-breakfast (all p>0.05, **Table 2**). There was a main effect of time (p<0.001, F=11.411), but not treatment (p=0.674, F=0.185) nor treatment x time interaction (p=0.681, F=0.222) for postprandial combined appetite score. Further, there was a main effect of time (p<0.03), but not treatment nor treatment x time interaction (all p>0.05), for hunger, fullness, satisfaction and prospective consumption during the postprandial period. There was no difference in volitional energy intake (p=0.736) in CON (1728 ± 681 kcal) compared to PRO (1666 ± 766 kcal).

[Table 2]

Sleep quantity and quality

There was no difference in self-reported sleep quantity between conditions (450 ± 52 v 444 ± 52 min, p=0.788). Reductions >10 mm were observed in the sleep quality measures of wakefulness (15 mm) and calmness (12 mm) in the PRO versus CON condition. However, there was no correlation between the difference in iAUC between conditions and the change in sleep wakefulness (r=0.017, p=0.953) or calmness (r=0.176, p=0.546) between trials.

DISCUSSION

This is the first study to investigate nocturnal protein ingestion in relation to the second-meal effect at breakfast. Contrary to our hypothesis, the primary findings demonstrate impaired postprandial glucose tolerance at breakfast after nocturnal protein ingestion, evidenced by the increased glycaemic response (iAUC) and peak blood glucose concentrations. Pre-breakfast (fasted) lipid oxidation was also elevated following protein ingestion, whilst postprandial after breakfast energy expenditure was higher. There was no difference in appetite or volitional energy intake between conditions. These findings illustrate that protein elicited a response opposite to the second-meal effect, impairing sequential glucose tolerance, with no effect on appetite or energy intake. This is the opposite response to that reported in the available literature regarding daytime meals during waking hours[15; 16; 18; 20].

The differences in glucose tolerance observed in the present study may be due to the 63 g dose of protein, as the majority of studies demonstrating a beneficial effect of a protein preload have used doses in the range of 10-55 g[19; 20; 21]. Further, previous studies have most commonly utilised a protein preload 30 min prior to the second meal [19; 20; 21], which differs from 5 h 39 min separating meals in the present study. Although both Meng *et al.* [24] and Park *et al.* [25] demonstrated that protein stimulates the second-meal effect with 4 h separating meals, both studies used high-protein breakfast foods, rather than the addition of whey protein used in the aforementioned preload studies[19; 20; 21]. Moreover, these studies were conducted in adults with type 2 diabetes[25] and postmenopausal women[24]. Regarding appetite, our results support the findings of Allerton *et al.* [28], which also reported no attenuation in appetite when whey protein was added to breakfast[28] ingested with a longer duration (i.e. 3 h) between meals than used in other preload studies[19; 20; 21].

Protein ingestion in excess of physiological needs can stimulate ureagenesis and the use of glucogenic amino acids in gluconeogenesis[40]. Boden and Tappy [41] demonstrated the hyperaminoacidemia following protein ingestion stimulated endogenous glucose production despite hyperinsulinemia. Studies employing stable isotope methodologies have demonstrated ~17-19% of protein ingested is converted to glucose[42; 43]. Therefore, assuming similar rates in the current study, it could be hypothesised that 17-19% of the 63 g protein ingested in the current study resulted in glucose production, producing 10.7 – 12.0 g glucose. This is supported by the findings of Ang *et al.* [44], who demonstrated that a 75 g of whey protein dose resulted in 11 g endogenous glucose production. Fromentin *et al.* [42] state that the percentage of dietary amino acids converted to glucose was mainly related to the provision of glucogenic amino acids. The whey protein utilised in this study comprised 79.8/100 g glucogenic or mixed amino acids (**Figure 1**), providing a high availability of gluconeogenic precursors. In addition, the relative lack of other sources of gluconeogenic precursors

(glycerol, pyruvate, lactate) in our whey protein solution, comparative to egg[42] or cottage cheese[43], may have further increased the contribution of amino acids to gluconeogenesis. Hence, glucose production may have been at the higher end of this estimate.

There are multiple underlying mechanisms through which the above-reasoned endogenous glucose production could alter glucose control following breakfast. Firstly, it may be that there was still some residual endogenous glucose production from the nocturnal protein by the time breakfast was ingested. Indeed, Ang *et al.* [44] reported that endogenous glucose production was still elevated at 4 h following whey protein ingestion, which could therefore supplement the carbohydrate directly ingested at breakfast and thus explain the elevated postprandial glycaemia in the PRO condition. It should be noted, however, that the breakfast in the present study was provided almost 6 h following the protein dose, so the contribution of the ingested protein towards endogenous glucose production may have subsided by the time of the second delivery of ingested nutrients.

Secondly, any glucose generated from the ingested amino acids even prior to breakfast could still contribute to a more positive carbohydrate balance (i.e. increased glycogen availability) and thus potentially limit the capacity for further non-oxidative carbohydrate disposal at breakfast. Certainly the 10-12 g of glucose that would theoretically arise via gluconeogenesis from the ingested protein could make a meaningful contribution towards the total capacity for hepatic glycogen stores. The current study employed a somewhat unusual protocol in that a large provision of energy was delivered at an atypical time of day when neither required nor expected by the body. Overnight fasting depletes hepatic glycogen stores and stimulates gluconeogenesis[45; 46], hence the liver may have been more sensitive to the large protein dose ingested during the night. It is unlikely that any large proportion of glucose produced would be oxidised in this rested and fasted state and therefore is more likely to be stored, increasing hepatic glycogen stores. An increase in glycogen stores may reduce the liver's capacity for first pass extraction following breakfast, reducing hepatic glucose uptake and resulting in more glucose remaining in circulation[47]. In the present study, the mean time between protein ingestion and breakfast was 5 h 39 min. Peak liver glycogen concentration is achieved around 5 h postprandially[46], therefore it is possible this peak may have coincided with breakfast, reducing the capacity for first pass extraction. However, rather than liver glycogen storage, the primary driver of postprandial glucose tolerance is thought to be insulin-stimulated glucose uptake into peripheral tissues and the synthesis of skeletal muscle glycogen[48; 49], yet 10-12 g of newly synthesised glucose would represent a much smaller contribution to whole-body muscle glycogen reserves and thus less likely to impair skeletal muscle glucose uptake at breakfast. The present results regarding glycaemic control therefore warrant future studies to examine such mechanisms by measuring glucose synthesis/disposal, along with muscle and liver glycogen concentrations.

The time of feeding in the present study may also have impacted gluconeogenesis and insulin sensitivity. Protein was fed at a time when the relative concentrations of both glucagon and cortisol were increasing. Elevated glucagon concentration stimulates hepatic gluconeogenesis and amino acid uptake[50; 51], while cortisol also stimulates hepatic gluconeogenesis, alongside reducing insulin sensitivity[50]. Betts *et al.* [52] demonstrated that the overnight cortisol response is elevated following the co-ingestion of protein with CHO before bed. Thus, the protein ingestion in the present study may have augmented the natural circadian elevation in cortisol thus reducing insulin sensitivity. There is also evidence for circadian rhythmicity in circulating amino acids, with lowest concentrations between 0400 and 0800 h[53; 54]. Feigin *et al.* [54] demonstrated that the ingestion of a large protein bolus at 0800 h resulted in an additional increase in blood amino acid concentrations, exceeding that anticipated from typical circadian periodicity, an interference not replicated with an identical protein load consumed at 2000 h. This further supports the idea that the body may not be entrained to deal with a large bolus protein dose at the time of feeding in the present study. Finally, whey protein ingestion and an increase in plasma branched-chain amino acids (BCAA), particularly leucine, have also been demonstrated to impair insulin sensitivity directly, especially in high doses [55; 56; 57]. The high BCAA (22.6/100 g) and specifically leucine content (10.6/ 100 g) of the whey protein (**Figure 1**) may therefore have impaired glucose uptake via that mechanism. As such, any priming effect of insulin secretion following protein ingestion may have been insufficient to overcome this reduced insulin sensitivity at breakfast. Therefore, the arrival of a large dose of amino acids at an atypical time of day might, via the hypothesised influence on hepatic gluconeogenesis and insulin sensitivity, result in impaired glucose control upon waking in a manner specific to this time-frame. This further highlights the novel nature of the current study employing an atypical feeding time. Additional study is warranted to investigate if the glucose tolerance to a second meal is impaired following protein ingestion prior to other daily meals in a more 'conventional' feeding pattern.

Pre-breakfast lipid oxidation was elevated in the PRO trial and post-prandial energy expenditure was also then higher. It should be noted, however, that these calculations were not adjusted based on 24-h nitrogen excretion and so assume negligible protein oxidation, which may not be a valid assumption in this experiment[37]. Witard *et al.* [58] reported that ingesting 40 g whey protein isolate stimulates phenylalanine oxidation for 4 h, so the 63 g ingested in the present study is likely to have stimulated a similar response, although this may have reduced by the time measurements were made 6 h later at breakfast. Nonetheless, if protein undergoes gluconeogenesis and is oxidised, then the respiratory quotient for that process is ~0.8 (the same as for direct protein oxidation), whereas the respiratory quotient if the newly synthesised glucose is stored is 0.4[59]. Any persistent protein oxidation or gluconeogenesis 6 h after the nocturnal bolus would therefore be expected to slightly

reduce the whole-body RER based on measured oxygen uptake and carbon dioxide production. However, the fact that the RER under both conditions were very similar and in the range of 0.85-0.89 means any such difference is likely to be small and thus it remains a reasonable alternative explanation that the ingested protein did in fact increase lipid oxidation.

In conclusion, consumption of a whey protein solution during the night impaired postprandial glucose control at breakfast, whilst increasing postprandial energy expenditure, with no effect on satiety or energy intake. As such, this approach would not be recommended to improve postprandial glucose control following breakfast. This paradoxical second-meal phenomenon may relate to an influence of protein oxidation on the availability of hepatic and/or skeletal muscle glycogen and/or insulin sensitivity.

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Conflict of Interest

ESS, EA, KC, JH, GP, and HAS declare no conflict of interest. JTG has received research funding and/or has acted as a consultant for Arla Foods Ingredients, Lucozade Ribena Suntory, Kenniscentrum Suiker and Voeding, and PepsiCo. JAB has received research funding and/or has acted as a consultant for GlaxoSmithKline, Lucozade Ribena Suntory, Kellogg's, Nestlé and PepsiCo.

Authorship

JAB and JTG formulated the research question, ESS, EA, KC, JH, GP, JTG and JAB designed the study, ESS, EA, KC, JH and GP collected the data, ESS analysed the data, ESS, HAS, JTG and JAB contributed to writing the manuscript and all authors approved the final version of the manuscript.

TABLES

Table 1. Pre-breakfast and postprandial (120 min) values for substrate utilisation, RER and energy expenditure. At pre-breakfast n = 13 due to difficulties associated with expired air collection. *significant difference between CON v PRO.

	CONTROL	PROTEIN	*p*
Pre-breakfast			
RER	0.88 ± 0.06	0.85 ± 0.05	0.102
CHO oxidation (g•min^{-1})	0.21 ± 0.10	0.18 ± 0.09	0.173
Lipid oxidation (g•min^{-1})	0.06 ± 0.02	0.07 ± 0.02	**0.045***
Energy expenditure (kcal•day^{-1})	1901 ± 375	1952 ± 302	0.196
Postprandial (120 min period)			
RER	0.89 ± 0.03	0.89 ± 0.05	0.804
CHO oxidation (g•120 min^{-1})	28.17 ± 7.37	31.88 ± 12.29	0.084
Lipid oxidation (g•120 min^{-1})	6.85 ± 2.11	6.58 ± 2.75	0.694
Energy expenditure (kcal•120 min^{-1})	168 ± 27	179 ± 33	**0.018***

Carbohydrate (CHO), respiratory exchange ratio (RER). Values expressed as mean ± standard deviation.

Table 2. Pre-breakfast (0 min) and postprandial (60, 120 min) subjective appetite responses.

Variable	0 min		60 min		120 min	
	CON	PRO	CON	PRO	CON	PRO
Hunger	42 ± 29	42 ± 28	27 ± 15	28 ± 21	40 ± 23	42 ± 23
Fullness	34 ± 29	32 ± 31	60 ± 21	59 ± 23	54 ± 23	53 ± 23
Satisfaction	38 ± 27	36 ± 28	63 ± 14	54 ± 24	52 ± 24	51 ± 24
Prospective consumption	55 ± 28	55 ± 28	33 ± 16	43 ± 20	47 ± 26	55 ± 26
Combined appetite score	57 ± 26	57 ± 24	34 ± 15	40 ± 18	45 ± 23	48 ± 20

Control (CON), protein (PRO). All variables demonstrated a main effect of time (p<0.03). Values expressed as mean ± standard deviation.

REFERENCES

1. Fletcher J, Perfield I, JW TJ *et al.* (2012) The second meal effect and its influence on glycemia. *Journal of Nutritional Disorders & Therapy* **2012**.
2. Borch-Johnsen K, Neil A, Balkau B *et al.* (1999) Glucose tolerance and mortality: Comparison of WHO and American Diabetes Association diagnostic criteria. The DECODE study group. European Diabetes Epidemiology Group. Diabetes Epidemiology: Collaborative analysis Of Diagnostic criteria in Europe. *Lancet* **354**, 617-621.
3. (2015) Diabetes Prevalence. In *Diabetes UK*.
4. Zafar TA, Waslien C, AlRaefaei A *et al.* (2013) Whey protein sweetened beverages reduce glycemic and appetite responses and food intake in young females. *Nutrition research* **33**, 303-310.
5. WHO (2006) Definition and diagnosis of diabetes mellitus and intermediate hyperglycaemia: report of a WHO/IDF consultation.
6. Ning F, Zhang L, Dekker JM *et al.* (2012) Development of coronary heart disease and ischemic stroke in relation to fasting and 2-hour plasma glucose levels in the normal range. *Cardiovascular Diabetology* **11**, 76.
7. (2018) Statistics on Obesity, Physical Activity and Diet - England, 2018 [PAS]. In *NHS Digital*.
8. Gonzalez JT, Stevenson EJ (2014) Calcium co-ingestion augments postprandial glucose-dependent insulinotropic peptide 1-42 , glucagon-like peptide-1 and insulin concentrations in humans. *European Journal of Nutrition* **53**, 375-385.
9. Fonseca V (2003) Clinical significance of targeting postprandial and fasting hyperglycemia in managing type 2 diabetes mellitus. *Current medical research and opinion* **19**, 635-631.
10. van Dijk J-W, Manders RJF, Hartgens F *et al.* (2011) Postprandial hyperglycemia is highly prevalent throughout the day in type 2 diabetes patients. *Diabetes Research and Clinical Practice* **93**, 31-37.
11. De Castro JM (2004) The time of day of food intake influences overall intake in humans. *The Journal of Nutrition* **134**, 104-111.
12. Hamman L, Hirschmann II (1919) Studies on blood sugar. Effects upon the blood sugar of the repeated ingestion of glucose. *Contributions to Medical and Biological Research* **2**, 306-308.
13. Astbury NM, Taylor MA, Macdonald IA (2011) Breakfast consumption affects appetite, energy intake, and the metabolic and endocrine responses to foods consumed later in the day in male habitual breakfast eaters. *The Journal of Nutrition* **141**, 1381-1389.
14. Gonzalez JT (2014) Paradoxical second-meal phenomenon in the acute postexercise period. *Nutrition* **30**, 961-967.
15. Ando T, Nakae S, Usui C *et al.* (2018) Effect of diurnal variations in the carbohydrate and fat composition of meals on postprandial glycemic response in healthy adults: A novel insight for the second-meal phenomenon. *The American Journal of Clinical Nutrition* **108**, 332-342.
16. Liljeberg HGM, Åkerberg AKE, Björck IME (1999) Effect of the glycemic index and content of indigestible carbohydrates of cereal-based breakfast meals on glucose tolerance at lunch in healthy subjects. *American Journal of Clinical Nutrition* **69**, 647-655.
17. Nilsson A, Granfeldt Y, Ostman E *et al.* (2006) Effects of GI and content of indigestible carbohydrates of cereal-based evening meals on glucose tolerance at a subsequent standardised breakfast. *European Journal Of Clinical Nutrition* **60**, 1092-1099.
18. Wolever TM, Bentum-Williams A, Jenkins DJ (1995) Physiological modulation of plasma free fatty acid concentrations by diet: metabolic implications in nondiabetic subjects. *Diabetes Care* **18**, 962-970.
19. Abou-Samra R, Keersmaekers L, Brienza D *et al.* (2011) Effect of different protein sources on satiation and short-term satiety when consumed as a starter. *Nutrition Journal* **10**, 139.
20. Akhavan T, Luhovyy BL, Brown PH *et al.* (2010) Effect of premeal consumption of whey protein and its hydrolysate on food intake and postmeal glycemia and insulin responses in young adults. *The American Journal of Clinical Nutrition* **91**, 966-975.

21. Ma J, Stevens JE, Cukier K *et al.* (2009) Effects of a protein preload on gastric emptying, glycemia, and gut hormones after a carbohydrate meal in diet-controlled type 2 diabetes. *Diabetes Care* **32**, 1600-1602.
22. Pal S, Ellis V (2010) The acute effects of four protein meals on insulin, glucose, appetite and energy intake in lean men. *British Journal of Nutrition* **104**, 1241-1248.
23. Bowen J, Noakes M, Clifton P (2007) Appetite hormones and energy intake in obese men after consumption of fructose, glucose and whey protein beverages. *International journal of obesity* **31**, 1696-1703.
24. Meng H, Matthan NR, Ausman LM *et al.* (2017) Effect of prior meal macronutrient composition on postprandial glycemic responses and glycemic index and glycemic load value determinations. *The American Journal of Clinical Nutrition* **106**, 1246-1256.
25. Park Y-M, Heden TD, Liu Y *et al.* (2015) A high-protein breakfast induces greater insulin and glucose-dependent insulinotropic peptide responses to a subsequent lunch meal in individuals with type 2 diabetes. *The Journal of Nutrition* **145**, 452-458.
26. Hall WL, Millward DJ, Long SJ *et al.* (2003) Casein and whey exert different effects on plasma amino acid profiles, gastrointestinal hormone secretion and appetite. *British Journal of Nutrition* **89**, 239-248.
27. Anderson G, Moore S (2004) Dietary proteins in the regulation of food intake and body weight in humans. *Journal of Nutrition* **134**, 974S-979S.
28. Allerton DM, Campbell MD, Gonzalez JT *et al.* (2016) Co-ingestion of whey protein with a carbohydrate-rich breakfast does not affect glycemia, insulinemia or subjective appetite following a subsequent meal in healthy males. *Nutrients* **8**, 116.
29. Jovanovic A, Leverton E, Solanky B *et al.* (2009) The second-meal phenomenon is associated with enhanced muscle glycogen storage in humans. *Clinical science* **117**, 119-127.
30. Pulido JME, Salazar MA (1999) Changes in insulin sensitivity, secretion and glucose effectiveness during menstrual cycle. *Archives of Medical Research* **30**, 19-22.
31. Horne JA, Östberg O (1976) A self-assessment questionnaire to determine morningness-eveningness in human circadian rhythms. *International Journal of Chronobiology* **4**, 97-110.
32. Knutson KL (2007) Impact of sleep and sleep loss on glucose homeostasis and appetite regulation. *Sleep Medicine Clinics* **2**, 187-197.
33. Kokkinos A, le Roux CW, Alexiadou K *et al.* (2010) Eating slowly increases the postprandial response of the anorexigenic gut hormones, peptide YY and glucagon-like peptide-1. *The Journal of Clinical Endocrinology & Metabolism* **95**, 333-337.
34. Nilsson A, Östman E, Granfeldt Y *et al.* (2008) Effect of cereal test breakfasts differing in glycemic index and content of indigestible carbohydrates on daylong glucose tolerance in healthy subjects. *American Journal of Clinical Nutrition* **87**, 645-654.
35. Wolever TMS (2004) Effect of blood sampling schedule and method of calculating the area under the curve on validity and precision of glycaemic index values. *British Journal of Nutrition* **91**, 295-300.
36. Péronnet F, Massicotte D (1991) Table of nonprotein respiratory quotient: an update. *Canadian Journal of Sport Sciences* **16**, 23-29.
37. Frayn KN (1983) Calculation of substrate oxidation rates in vivo from gaseous exchange. *Journal of applied physiology: Respiratory, environmental and exercise physiology* **55**, 628-634.
38. Zisapel N, Nir T (2003) Determination of the minimal clinically significant difference on a patient visual analog sleep quality scale. *Journal of Sleep Research* **12**, 291-298.
39. Cohen J (1988) *Statistical power analysis for the behavioral sciences.* New York: Routledge.
40. Schutz Y (2011) Protein turnover, ureagenesis and gluconeogenesis. *International Journal for Vitamin and Nutrition Research* **81**, 101-107.
41. Boden G, Tappy L (1990) Effects of amino acids on glucose disposal. *Diabetes* **39**, 1079-1084.
42. Fromentin C, Tomé D, Nau F *et al.* (2013) Dietary proteins contribute little to glucose production, even under optimal gluconeogenic conditions in healthy humans. *Diabetes* **62**, 1435-1442.

43. Khan MA, Gannon MC, Nuttall FQ (1992) Glucose appearance rate following protein ingestion in normal subjects. *Journal of the American College of Nutrition* **11**, 701-706.
44. Ang T, Bruce CR, Kowalski GM (2019) Postprandial aminogenic insulin and glucagon secretion can stimulate glucose flux in humans. *Diabetes* **68**, 939-946.
45. Ahlborg G, Felig P, Hagenfeldt L *et al.* (1974) Substrate turnover during prolonged exercise in man. Splanchnic and leg metabolism of glucose, free fatty acids, and amino acids. *Journal of Clinical Investigation* **53**, 1080-1091.
46. Gonzalez JT, Fuchs CJ, Betts JA *et al.* (2016) Liver glycogen metabolism during and after prolonged endurance-type exercise. *American Journal of Physiology Endocrinology and Metabolism* **311**, E543-553.
47. Jensen J, Rustad P, Kolnes A *et al.* (2011) The role of skeletal muscle glycogen breakdown for regulation of insulin sensitivity by exercise. *Frontiers In Physiology* **2**.
48. Meyer C, Dostou JM, Welle SL *et al.* (2002) Role of human liver, kidney, and skeletal muscle in postprandial glucose homeostasis. *American Journal of Physiology-Endocrinology And Metabolism* **282**, E419-E427.
49. Jackson R, Roshania R, Hawa M *et al.* (1986) Impact of glucose ingestion on hepatic and peripheral glucose metabolism in man: an analysis based on simultaneous use of the forearm and double isotope techniques. *The Journal of Clinical Endocrinology & Metabolism* **63**, 541-549.
50. Bodo RCD, Altszuler N (1958) Insulin hypersensitivity and physiological insulin antagonists. *Physiological Reviews* **38**, 389-445.
51. Frayn KN (2010) *Metabolic Regulation.* Oxford: Wiley-Blackwell.
52. Betts JA, Beelen M, Stokes KA *et al.* (2011) Endocrine responses during overnight recovery from exercise: impact of nutrition and relationships with muscle protein synthesis. *International journal of sport nutrition and exercise metabolism* **21**, 398-409.
53. Feigin RD, Klainer AS, Beisel WR (1967) Circadian periodicity of blood amino-acids in adult men. *Nature* **215**, 512-514.
54. Feigin RD, Klainer AS, Beisel WR (1968) Factors affecting circadian periodicity of blood amino acids in man. *Metabolism* **17**, 764-775.
55. Smith GI, Yoshino J, Stromsdorfer KL *et al.* (2015) Protein ingestion induces muscle insulin resistance independent of leucine-mediated mTOR activation. *Diabetes* **64**, 1555-1563.
56. Würtz P, Soininen P, Kangas AJ *et al.* (2013) Branched-chain and aromatic amino acids are predictors of insulin resistance in young adults. *Diabetes Care* **36**, 648-655.
57. Flakoll P, Wentzel L, Rice D *et al.* (1992) Short-term regulation of insulin-mediated glucose utilization in four-day fasted human volunteers: Role of amino acid availability. *Diabetologia* **35**, 357-366.
58. Witard OC, Jackman SR, Breen L *et al.* (2014) Myofibrillar muscle protein synthesis rates subsequent to a meal in response to increasing doses of whey protein at rest and after resistance exercise. *The American journal of clinical nutrition* **99**, 86-95.
59. Jéquier E, Acheson K, Schutz Y (1987) Assessment of energy expenditure and fuel utilization in man. *Annual Review of Nutrition* **7**, 187-208.

592 **FIGURE LEGENDS**

593 **Figure 1. Amino acid profile of the whey protein used.**

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595 **Figure 2. Protocol schematic. A mixed macronutrient tolerance test was conducted following**
596 **nocturnal ingestion of either protein (PRO) or water (CON).**

597

598 **Figure 3. A) Peak blood glucose concentration (conc.) and B) blood glucose iAUC. Dashed lines**
599 **denote female (n = 7) and solid lines denote male (n = 7) participants. Darker lines represent**
600 **individuals with a BMI >25 kg.m^{-2} (n = 4) and lighter lines denote those with a BMI <25 kg.m^{-2}**
601 **(n = 10). *significant difference between CON v PRO trials.**

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603 **Figure 4. Blood glucose concentration following breakfast ingestion at 0 min. The dashed line**
604 **represents a blood glucose concentration of 7.5 mmol·L^{-1} as a reference threshold.**

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